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PRELIMINARY STUDY ON *IN VITRO* PROPAGATION OF *Macaranga tanarius* (Mahang)

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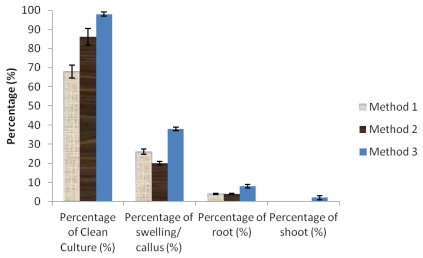
Graphical abstract



Abstract

Macaranga tanarius locally known as Mahang, belongs to Euphorbiaceae family is a native plant in Malaysia. This invasive species is mostly found in disturbed forest. *Macaranga* genus is a medium size tree and can grow up to 24 m tall. *Macaranga* genus is a fast growing species, evergreen trees with soft and light wood. *M. tanarius* wood can be used to produce high quality particle board and pulp besides other usage as good firewood. Phytochemical studies on *M. tanarius* have discovered the presence of tannin that can be used as toughing agent on fishing net as well as dyeing agent. The leaves extract of this species have shown potential as an antioxidant when tested with 2, 2 – diphenyl-picrylhydrazyl (DPPH) anti-oxidant assay. Due to its benefits in multiple industries, production of sustainable and high quality planting material cannot be avoided. Tissue culture is one of the best approaches to meet this demand. In this preliminary study, surface sterilization protocol for *M. tanarius* using seed as explants has been developed. 3 different surface sterilization methods have been tested. Based on the percentage of contamination and response, the best method for surface sterilization of *M. tanarius* is by using 30% of Chlorox® which produced more than 90% clean culture and the best response among other methods; swelling (38%), formation of roots (8%), shoots (2%) as well as minimum amount of damage tissues. Explants from germinated plantlets *in vitro* were further tested on four different basal medium to find the most suitable basal medium for *M. tanarius* growth which were full strength Woody Plant Media (WPM), half strength Woody Plant Media (½ WPM), Murashige and Skoog media (MS) and half strength Murashige and Skoog media (½ MS). Explants cultured on WPM basal medium produced healthy rooted plantlet in terms of size and colour of shoot and leaves. ½ WPM media can also induce rooting in *M. tanarius* whereas in MS and ½ MS media the explants turn to brown and died. For shoot multiplication experiment, WPM medium supplemented with different types of cytokinin; BAP and TDZ at different concentration have been tested. WPM medium supplemented with 0.1 mg/l TDZ showed the highest percentage of shoot induction with 100% shoot induced and average produces three new shoots per explants.

Keywords: *Macaranga tanarius*, *in vitro* propagation

1.0 INTRODUCTION

Macaranga tanarius locally known as "Mahang" has been used as a plant sample for this research. *Macaranga* species which belongs to Euphorbiaceae famil, is a soft wooded tree and can grow up to 24 m tall. The leaves are peltate, ovate to oblong-ovate, 8 – 30 cm long and have tiny hairs on its surface while its twigs and stalks have smooth surface. The side leaves on small and mature tree are around 35 cm and 7.5-23 cm long and the petiole is about 5-20 cm long with a bunch of flowers at 10 -20 cm of the petiole. The armpits leaves are pale green colour and the stem vein is usually pink. As for its inflorescence, male has more branch than females. The fruits consist of muricated capsule, which encapsulating 2 or 3 seeds together, have soft hair and a sticky white/yellow powder at its surface (Figure 1). The fruit size is about 0.6-1.2 cm long and 1.2 cm wide [1; 2; 3; 4].

M. tanarius can be used to produce high-quality pulp and particleboard. The timber is fairly tough even it is not durable or resistant to termite attack, has straight or only shallowly interlocked grain, with a moderately fine and even texture. *M. tanarius* has been recommended as a shade tree to provide shelter for other crops and as firewood [2; 3; 5).

Besides wood physical character of *M. tanarius*, phytochemical content in different parts of the plant were also being studied. Extracts from different parts of the plant were being consumed as traditional medication for anti-pyretic, anti-tussive, emetic agent and anti-inflammation [6; 7]. Anti-oxidant, prenylflavanoid and megastigmane glucosides were found in the leaves extract along with macaflavanones A–G, compounds from flavanones group. The anti-oxidant prenylflavanoid was also detected in flower, seed, pericarp and glandular trichome of female *M. tanarius* [8; 9]. The macaflavanones A–G showed cytotoxicity against two cell lines [10]. Tannin was also found in *M. tanarius* bark and used to toughening fishing net [2].

The discovery of *M. tanarius* potential usage especially in timber and pharmacological industries will induce demand for its raw material. Preparation of good quality and sustainable raw material is important in commercialization. Hence, suitable propagation method for *M. tanarius* needs to be developed. So far, the propagation of this species was through seed [5; 11] and no propagation through tissue culture has been reported. In this study, the protocol for propagation of *M. tanarius* in tissue culture has been developed.

Figure 1 *M. tanarius* seeds and leaves

2.0 EXPERIMENTAL

2.1 Surface Sterilization of Seed

Fruits of *M. tanarius* were collected from Bukit Bujang, FRIM. The fruits were surface sterilized with 70% ethanol and commercial bleach, Chlorox® at 3 different concentrations (Method 1: 50%, Method 2: 40% and Method 3: 30%) for 15 minutes. *M. tanarius* fruit consists of muricated capsule and this capsule were opened and removed to release 2 to 3 seeds from each fruit. The seeds were cultured on WPM basal media and kept in growth chamber at 22 ± 2°C. The percentage of clean culture and germination were recorded after 8 weeks in culture.

2.2 Basal Medium

WPM [12] and MS [13] basal medium (stock powder) were obtained from Duchefa Biochemie (WPM Product No: M0220.0050; MS Product No; M0222.0050). 2 different basal media at different strength were prepared to determine the suitable medium for *M. tanarius* culture; full strength WPM basal, full strength MS basal, half strength WPM (½ WPM) basal and half strength MS (½ MS) basal media. All media was solidified with 3% of gelrite and 30 g/L of sucrose as carbon source. The pH was adjusted to 5.8 prior to autoclaving at 121°C for 15 minutes. *M. tanarius* cultures were maintained at 22 ± 2°C with 16 h light and 8 h dark. The morphology of explants in each medium was observed and recorded after 8 weeks.

2.3 Medium Preparation for Shoot Multiplication

Full strength WPM basal medium supplemented with benzyl-amino-purine (BAP: 0 mg/l, 0.1 mg/l, 0.25 mg/l, 0.5 mg/l, 1.0 mg/l, 2.0 mg/l) and thidiazuron (TDZ: 0 mg/l, 0.1 mg/l, 0.25 mg/l, 0.5 mg/l, 1.0 mg/l, 2.0 mg/l) were tested for shoot induction and multiplication. WPM basal medium without an addition of plant growth regulator were used as control. Each treatment was repeated three times. All media were solidified with 3% of gelrite and 30 g/L of sucrose as carbon source. The pH was adjusted to 5.8 prior to

sterilization at 121°C for 15 minutes. Cultures were maintained at 22 ± 2°C with 16 h light and 8 h dark. Number of new shoots, height, callus and root formation were observed and recorded after 8 weeks.

2.4 Statistical Analysis

Data on the effects of plant growth regulators on shoot multiplication was analysed by one-way analyses of variance (ANOVAs) using SAS (version 9.1.3). Values are means of four replicates and repeated three times, and the presented mean values were separated using Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$

3.0 RESULTS AND DISCUSSION

3.1 Seed Surface Sterilization

Seeds of *M. tanarius* were used as source of explants materials. Different surface sterilization methods were used to determine the most suitable method for *M. tanarius* seeds according to the percentage of clean culture and germination. 3 different concentrations of commercial bleach, Chlorox® were tested. Method 3: 30% Chlorox® showed the highest percentage of clean culture and responses (Figure 2). After 8 weeks in culture, the seeds started to germinate. Some of the seeds break their seed coats and produced masses of white callus before emergence of radical and developed into normal seedlings (Figure 3). White callus produced probably induced by cell injury during surface sterilization and culturing.

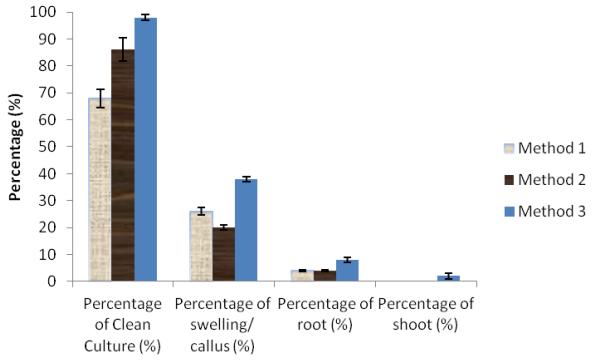


Figure 2 Effect of different surface sterilization methods on *M. tanarius* culture initiation

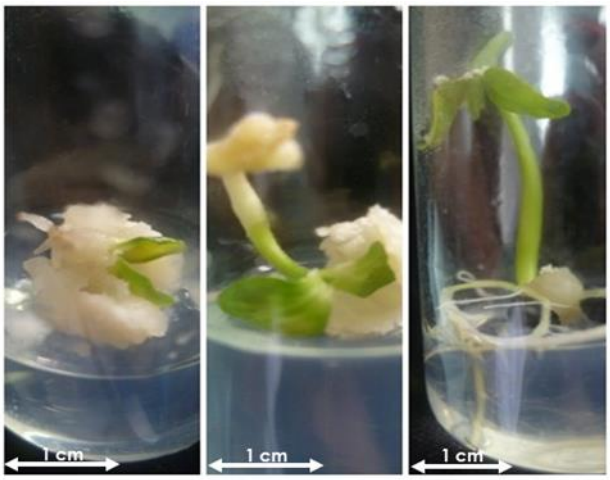


Figure 3 Germination of *M. tanarius* seeds after 8 weeks in culture

3.2 Basal Medium for *M. tanarius* Cultures from Seed Explants

Suitable medium for *M. tanarius* cultures need to be determined. From previous observation, *M. tanarius* explants become browning and died after eight weeks cultured in MS medium and showed no response on shoot multiplication. Due to this observation, 2 different basal media with different strength were tested. From the observation, WPM basal medium and its half strength showed positive responses on *M. tanarius* survivability based on the length of shoots and leaves as well as the leaves colour. Shoots in WPM basal and half strength WPM basal media rooted even without presence of rooting plant growth regulator. Between the two media, explants in full strength WPM basal medium were healthier but produced fewer root than in half strength WPM basal medium (Figure 4).

However, in full strength MS and half strength MS basal media, negative responses were observed where the growth of the explants was stunted, browning and died after eight weeks in culture. Based on the results, WPM basal media is more suitable for *M. tanarius* cultures instead of MS basal media. The difference in WPM basal medium and MS basal medium is the nutrient or salt concentration. Nutrient or salt content in the medium played major role for cell differentiation, growth, elongation and other metabolism processes and therefore is a crucial step in tissue culture. The concentration of salt and sugar determined the osmotic properties of the media [14]. MS basal medium is widely used and suitable for many plant species but not for *M. tanarius* based on the results. Comparing WPM basal and MS basal media, WPM basal contained lower salt, 25% of nitrate and ammonium ion but higher potassium and sulfate ion than MS basal media [15]. Similar research on woody plant, *Anacardium othonianum* [16], showed suitability in low salt media like WPM. WPM basal media also have shown success in

multiplication of other woody species such as *Eurycoma longifolia* [17] and *Shorea robusta* [18].

3.3 Shoot Multiplication

Full strength WPM basal medium was used for further experiment on shoot multiplication with supplementation of BAP and TDZ at concentrations of 0 mg/l, 0.1 mg/l, 0.25 mg/l, 0.5 mg/l, 1.0 mg/l and 2.0 mg/l.

Table 1 showed overall result obtained from shoot multiplication of *M. tanarius* in different plant growth regulators. Results showed that 0.1 mg/l, 0.5 mg/l and 1.0 mg/l BAP able to induce 50% of the explants to produce new shoots after 8 weeks in culture. However, number of shoots multiplied per explants was low, only about 1 new shoot per explant. It was also observed that WPM basal medium without plant growth regulator resulted in healthy plantlets but did not induced shoot multiplication. In media containing BAP, the explants were stunted, their leaves size was also small and light green colour indicating the explants were in stress condition.

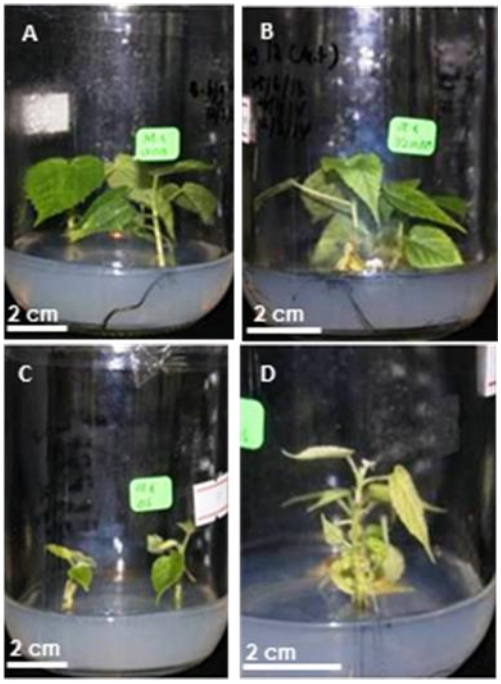


Figure 4 *M. tanarius* shoots in different basal medium after eight weeks in culture. a) WPM basal medium, b) Half strength WPM (1/2 WPM) basal medium, c) MS basal medium and d) Half strength MS (1/2 MS) basal medium

Due to unresponsiveness of *M.tanarius* cultures in medium containing BAP, TDZ was used at different concentrations. In medium containing TDZ, the highest number of shoot produced was 3 new shoots per explants with average height of 0.6 ±0.10 cm in 0.1 mg/l TDZ after 8 weeks in culture, followed by 0.25 mg/l TDZ with average 2 new shoots per explant. Full strength WPM basal media supplemented with 0.1 mg/l and 0.25 mg/l TDZ were able to induce 100% and 92% bud break.

Significant differences were found in number of new shoots produced for 0.1 mg/l and 0.25 mg/l TDZ and in height of new shoot for 0.1 mg/l TDZ. The new shoots produced were small with average 0.6 cm height, however the maximum new shoot's height can reached up to 1.0 cm with small leaves and light green leaves colour. The growth of the new shoots was slowed compared to the explants in medium without plant growth regulator.

The formation of non-friable callus was observed on most of the explants in media supplemented with cytokinin. The callus was greenish to brown in colour. Explants in full strength WPM basal medium without plant growth regulator does not show any formation of callus, contrary to the media supplemented with cytokinin, where all medium supplemented with BAP or TDZ showed callus formation. The percentage of callus formation in media supplemented with BAP can reached maximum of 33% in 0.5 mg/l BAP and 83% in media supplemented with 0.25 mg/l and 2.0 mg/l TDZ. The formation of callus probably affect the induction of root from explant in media supplemented with TDZ where none of the explant rooted in these media. However, in media containing BAP, even though formation of callus occurred, the percentage of rooted explants was not greatly affected where the highest percentage of rooted explants was 50% in media supplemented with 0.1 mg/l, 0.5 mg/l and 2.0 mg/l BAP. In media without plant growth regulator, 100% of explants rooted.

From these experiment, the suitable medium for shoot induction and multiplication of *M. tanarius* is full strength WPM basal medium supplemented with 0.1 mg/l TDZ. This result is similar to previous report on multiplication of *Salix nigra* Marsh, where WPM medium supplemented with TDZ supported the highest percentage of shoots and bud formation [19].

Table 1 Effect of WPM basal medium supplemented with different concentration of BAP and TDZ on shoot multiplication of *M. tanarius*

BAP (mg/L)	TDZ (mg/L)	Percentage of bud break(%)	No of new shoot*	New shoot height (cm)*	Height of shoots explants (cm)*	Percentage of callus formation (%)	Percentage of explants rooted (%)
-	-	-	0 ^{d}	0 ^{d}	2.025 ± 0.19 ^{ab}	-	100
0.1	-	50	1 ± 0.19 ^{cd}	0.275 ± 0.09 ^{bcd}	1.708 ± 0.185 ^{b}	25	50
0.25	-	7	0 ^{c}	0 ^{cd}	1.917 ± 0.352 ^{ab}	17	33
0.5	-	50	1 ± 0.29 ^{c}	0.317 ± 0.127 ^{cb}	2.55 ± 0.338 ^{a}	33	50
1	-	50	1 ± 0.19 ^{cd}	0.263 ± 0.085 ^{bcd}	1.392 ± 0.168 ^{b}	25	33
2	-	17	0 ^{cd}	0 ^{cd}	1.992 ± 0.275 ^{ab}	25	50
-	0.1	100	3 ± 0.25 ^{a}	0.600 ± 0.105 ^{a}	2.067 ± 0.123 ^{ab}	75	-
-	0.25	92	2 ± 0.26 ^{b}	0.435 ± 0.07 ^{ab}	2.067 ± 0.15 ^{ab}	83	-
-	0.5	-	1 ± 0.29 ^{cd}	0.254 ± 0.113 ^{bcd}	1.65 ± 0.131 ^{b}	50	-
-	1	33	1 ± 0.23 ^{cd}	0.167 ± 0.09 ^{bcd}	1.883 ± 0.123 ^{ab}	42	-
-	2	25	1 ± 0.29 ^{cd}	0.100 ± 0.05 ^{cd}	1.667 ± 0.112 ^{b}	83	-

*Values are mean ± S.E, means followed by the same letter are not significantly different ($p \leq 0.05$) according to Duncan's multiple range test.

However, since the new shoots produced were stunted and slow growth, elongation step was probably required, by subculturing the new shoots in WPM basal media without addition of plant growth regulators or low cytokinin concentration. TDZ is the most active cytokinin-like substances that have facilitates multiplication and shoot regeneration in many woody plant species. However, TDZ may inhibit shoot elongation, callus formation and slightly inhibit root formation as seen in this experiment [20]. According to Guo *et al.* (2011), TDZ have both cytokinin and auxin like effect on plant, even though it is chemically different with the two plant growth regulator [21]. High percentage of callus formation in *M. tanarius* was probably occured due to this character (Figure 5).

In this experiment, WPM basal medium was not only suitable for shoot elongation, but also can induce rooting. Therefore, rooting step can be avoided where cost will be much more lower and duration for transfering explants from growth chamber to nursery can be shortened.

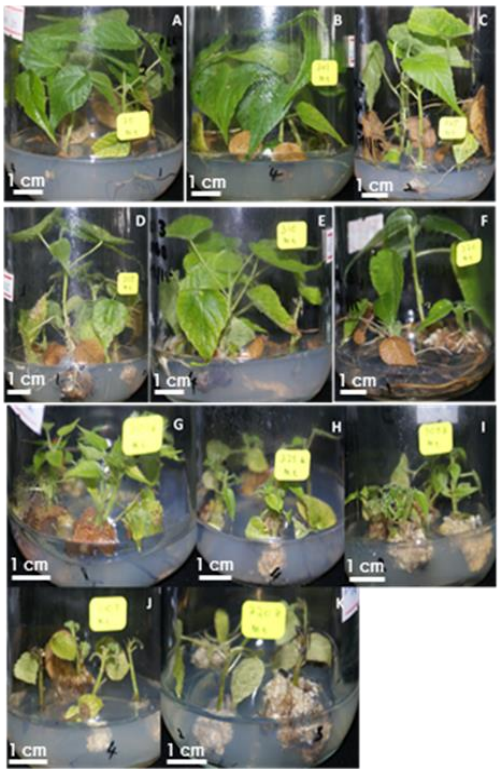


Figure 5 Shoot multiplication of *M. tanarius* in WPM basal medium supplemented with different concentration of BAP and TDZ A) WPM basal medium (Control) without plant growth regulator, B) WPM basal medium + 0.1 mg/l BAP, C) WPM basal medium + 0.25 mg/l BAP, D) WPM basal medium + 0.5 mg/l BAP, E) WPM basal medium + 1.0 mg/l BAP, F) WPM basal medium + 2.0 mg/l BAP, G) WPM basal medium + 0.1 mg/l TDZ, H) WPM basal medium + 0.25 mg/l TDZ, I) WPM basal medium + 0.5 mg/l TDZ, J) WPM basal medium + 1.0 mg/l TDZ, K) WPM basal medium + 2.0 mg/l TDZ

4.0 CONCLUSION

M. tanarius can be introduced into tissue culture by following protocol developed from this research since suitable surface sterilization method and multiplication medium have been determined. However, the next crucial step is plantlets acclimatization in nursery. The acclimatization experiment is required to complete *in vitro* propagation protocol of *M. tanarius.*

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